EMBRYO DORMANCY OF PINUS JEFFREYI MURR. SEED AS AFFECTED BY TEMPERATURE, WATER UPTAKE, STRATIFICATION, AND SEED COAT [1, 2]

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Almost three hundred years ago the English silviculturist, John Evlyn (7), recognized the general problem of forest tree seed dormancy. He did not discuss dormancy as such, but he did report that fall or early winter sowing of acorns, beech-nuts, haws and holly berries was a better practice than spring sowing. He went on to state that ". . . seeds may be prepared for the Vernal by being barrell'd or potted up in moist sand or earth stratum during the winter at the expiration whereof you will find them sprouted and being committed to the earth as apt to take as if they had been sown with the most early . . . and will not be much concern'd with the increasing heat of the season, as such as being crude, and unfermented are newly sown in the beginning of the Spring. . . ." Thus, here is perhaps the first published recommendation for artificial stratification of dormant tree seed. It is obvious he did not know why it was effective—as a matter of fact, we do not know why today, even though we have recognized some of the associated biochemical changes (6, 8, 11, 12, 14).

Since the turn of the century numerous studies on seed dormancy have been reported; see reviews by Miller (11), Baldwin (1), Crocker (4), and Crocker and Barton (5). Specific reference to pine seed indicates that the dormancy mechanism of that seed is not entirely clear. For example to overcome dormancy, at least five different pregermination treatments have been recommended. They are: soaking the seeds in sulfuric acid (17); soaking the seeds in water at room temperature (9); soaking the seeds in water at low temperature (15, 16); cold storage in wet sand or peat moss (2, 3, 13, 23) and removal of the seed coat and papery membrane (19).

The most widely used method of increasing seed germination is the low-temperature moist storage treatment generally referred to as stratification. During this particular type of pretreatment chemical changes apparently take place in the seed, after which the seed germinates. For example, Eckerson (6) found during stratification of *Crataegus mollis* seeds that the fats decreased while there was a progressive increase in sugars, acidity, catalase activity, peroxidase activity, and water-holding capacity. Similar results are reported by Pack (14) for seeds of *Juniperus virginiana, J. communis* and *J. prostrata.* He found that fats and proteins decreased while sugars, amino acids, catalase activity, and respiration all increased during stratification.

On the other hand Koblet (10) found little change in the character of the food reserve in *Pinus strobus* seeds during stratification. Only a slight increase in reducing sugars, amino acids, and soluble nitrogen was noted. From this he concluded that the low temperature treatment facilitated a faster mobilization of the reserves during germination and ". . . that low temperatures could effectively stimulate the plasma or upset its equilibrium thereby giving an impulse to growth."

The major change Mirov (12) found in stratified *Pinus lambertiana* seeds was an increased saturation of the fats. His data also suggested a build-up of auxin in the endosperm during stratification — a change which Haddock (8) subsequently was unable to detect.

Stratification is required by some but not all species of pine for satisfactory germination. The "Woody Plant Seed Manual" (21) lists thirteen native species of pine as not requiring stratification. Nevertheless, when seeds of many of these thirteen species are stratified they germinate more quickly and at a more uniform rate than when they are not stratified (2, 3, 13, 23). Therefore a difference in degree rather than a difference in kind is indicated in these two groups. The same metabolic pattern might well prevail in both groups of pine while the level of substrate, auxin, co-factors and inhibitors might be different.

The dormancy of a large number of temperate zone seeds that responded favorably to stratification can also be overcome by breaking or removing the seed coat. This has generally been interpreted as indicating non-dormant embryos, and the pines are often placed in this category (4, 5, 11, 23). However, Stone and Duffield (19) have reported that under certain conditions the embryo of *Pinus lambertiana* was at least partially dormant.

An attempt by the author in 1949 to evaluate conflicting reports by Mirov (12), Haddock (8), and Stone (18) regarding auxin change during stratification was only partially successful (unpublished data). Auxin changes did occur during stratification of *Pinus lambertiana* seed, but it varied from one seed lot to the next. It was obvious that "standardized" seed was needed. Therefore, the following year a large amount of seed was collected from one tree, dried to a moisture content of ten percent, and then stored at 5° C in sealed containers. However, even this uniformly treated seed could not be considered "standardized" because germination behavior changed considerably during the first twelve months of storage. Following this discovery it became important to obtain more specific information about germination behavior prior to the continuation of the auxin studies. Consequently a series of germination behavior studies was undertaken.

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During the ensuing five years a number of experiments were carried out, none of which were very important in themselves. Now, however, an interesting picture can be drawn from the overall data. Not only was the germination followed for *Pinus lambertiana* seed which normally requires stratification but also for *Pinus jeffreyi* which normally does not (21). Relevant parts of this study that deal with *Pinus jeffreyi* are reported here.

Materials and Methods

The seed used in this study was collected in 1952 from several trees located in northeastern California. After air drying for a week or more the seed was cleaned, sealed in five gallon cans, and shipped to Berkeley. There it was stored at 1° C until used in 1954 and 1955. The moisture content of the seed varied from 10 to 13 %, with an average of 12 %. Cutting tests showed that 4 % of the seeds were empty or otherwise morphologically defective. At the time of this study freshly harvested seed was not available. However, with *Pinus jeffreyi* this does not assume the importance it does with *Pinus lambertiana*. Seeds of *Pinus jeffreyi* store well at 1° C for several years without any appreciable reduction in its germination (21).

Germination techniques, sample size, and the selection of appropriate germination temperatures were based upon preliminary experiments. The germination temperatures selected were 5, 15 and 25° C, and in each case were held to within $\pm$ 0.5° C throughout the experiment. The samples were replicated ten times and were of ten seeds each.

As a measure of the effect of the various seed treatments upon germination at the different temperatures, the number of days required to reach 50 % germination was selected. The 50 % germination level was used rather than the 90 % or some other level, because germination was close to its maximum rate at this point and because the total germination of each seed sample used at least reached the 50 % level. For a statistical expression of the significance of the difference of germination at the 50 % germination level, the standard error (S.E.) of the difference of the means was calculated and the t-test applied.

In the water uptake experiments, samples of twenty-five seeds each, replicated five times were used. Although the S.E. of the mean was large the effect of the treatment, e.g., difference in water uptake after removal of the seed coat, was always many times greater and a statistical expression of the significance of the difference was not considered necessary.

Stratification: Two different methods of stratification were used. The first, or standard method consisted of mixing the seed with wet vermiculite, pouring the mixture into pint ice cream cartons, sealing the cartons and then storing them in a cold room at 5° C for three months.

The second method consisted of first soaking the seed for 48 hours in 5° C tap water through which air was continuously bubbled. Then the seed was removed, sealed in large test tubes, without vermiculite or other stratification media, and stored in a cold room at 5° C for three months.

Germination of Whole Seed: These studies were carried out using Petri dishes filled with wet vermiculite. Ten seeds were placed on the bottom of each dish before the vermiculite was added. It was thus possible to observe germination [3] without having to remove the seed from the germinating medium. The Petri dishes were stacked on shelves in the appropriate incubator and inspected daily. From time to time water was added when the surface of the vermiculite appeared dry. Any water not absorbed by the vermiculite was readily observed and drained off after each addition of water. Except for the short periods when measurements were being made, seeds were kept in the dark.

Germination Without the Seed Coat: In general the procedure was the same as outlined by Stone and Duffield (19). Seeds were surface sterilized by soaking for five minutes in bromine water, before being opened in a sterile transfer room. Then after the seed coat and papery membrane, derived from the inner portion of the integument, and the remains of the nucellus were removed, the embryo, still surrounded by the endosperm, was placed upon solidified 1 percent agar in 25 × 200 mm screw top culture tubes. These were then placed in the appropriate incubator and germination regularly observed. Again, except for the short periods when measurements were being made seeds were kept in the dark.

Some contamination did occasionally occur. However, since each seed was in a separate culture tube, contamination did not spread and in these studies was never of any consequence.

Water Uptake Measurements: These measurements were made with: (a) whole seed, (b) seed from which the seed coat and papery membrane had been removed, and (c) isolated embryos and endosperm tissue. In some experiments the seed was stratified, in others it was not, and in still others the seed was killed [4] by heating for one-half hour at 100° C in stoppered test tubes.

One of the methods used to follow water uptake was to place the seed in Erlenmeyer flasks half filled with water through which air was bubbled. Then every twenty-four hours samples of the seed were taken out, the seed coat removed and the moisture content of the combined endosperm and embryo tissues determined.

[3] Germination, as used in this paper, refers to the initial appearance of the radicle. Subsequent elongation of both the radicle and the hypocotyl are not in any way reflected in the germination data as is the case when germination is determined by the number of seedlings appearing above the ground. The need for this distinction will be developed in a subsequent paper.

[4] When seed was so treated the embryos, contrary to embryo behavior from untreated seed, did not show any growth activity when excised. Furthermore there was no measurable gas exchange in such seeds even after they had imbibed water.

Another method was used in following the water uptake of isolated embryos and endosperm tissue. The particular tissue was placed on a stack of water-saturated filter papers in the bottom of a Petri dish. Saturation of the paper was maintained by the repeated addition of sterile water and by keeping the cover on at all times except when weighings were being made. Individual seeds were weighed daily on a Roller-Smith balance in a sterile transfer room. Necessarily seeds were removed from the incubators for short periods of time. However, the maximum time any seed was out during any 24-hour period was 5 minutes. The steps associated with sterile removal of the seed coat as outlined earlier were also followed in the water imbibition studies with endosperm and embryos in order to reduce contamination and the attendant complications.

Results

Germination of Stratified and Unstratified Whole Seed: At 25° C, germination of the unstratified seeds reached the 50 % level in 23 days. On the other hand, when stratified, the 50 % level was reached in three days (fig 1).

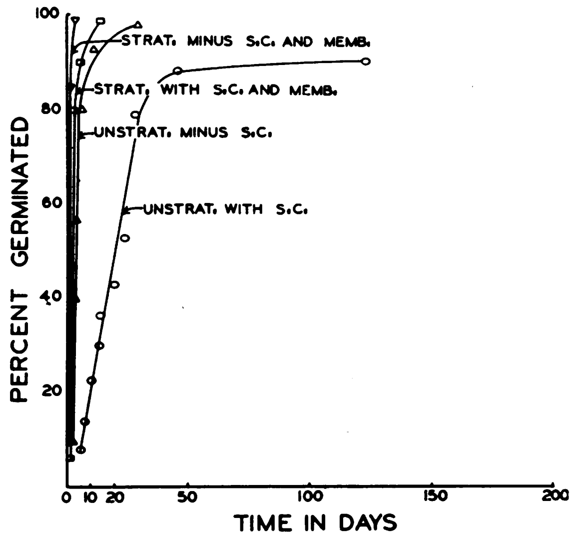
A ten-degree drop in the germination temperature had a marked effect upon the unstratified seeds, but little effect upon the stratified seeds. Fifty-percent germination of the unstratified seeds required 115 days while the stratified seeds reached the same level in only 6 days (fig 2).

The same general pattern was continued when the germination temperature was reduced to 5° C, although the order of magnitude was different. At this temperature unstratified seeds took 175 days for 50 % to germinate while stratified seed took only 50 days (fig 3). Thus, stratification reduced the germination time at the three temperatures used.

Germination after Removal of the Seed Coat and Papery Membrane: Removal of the seed coats and papery membranes from the unstratified seeds increased the germination rate at all three temperatures. At 25° C the number of days required for 50 % of the seeds to germinate was reduced from 23 to 4; at 15° C it was reduced from 115 to 14; and at 5° C it was reduced from 175 to 95 (figs 1 to 3). Thus here is a pattern similar to that achieved through stratification. However, although the patterns are similar, stratification is more effective in increasing the germination rate than is the mere removal of the seed coats and papery membranes. At 25° C, 50 % of the stratified seeds germinated in 2 days, but it took 4 days when unstratified seed were used and only the seed coats and papery membranes removed; at 15° C it took 6 days and 14 days respectively and at 5° C it took 50 days and 95 days respectively (figs 1 to 3).

When the seeds were stratified and the seed coats and papery membranes were also removed the most rapid germination was attained. Once again the effect of stratification was more evident at 5° C than at 15° C or 25° C. Even at 5° C germination was fairly rapid with 50 % of the germination completed in 10 days. In contrast stratified whole seeds took 50 days, unstratified seeds without seed coats and papery membranes took 95 days, and whole unstratified seeds took 175 days (figs 1 to 3).

These results summarized in figure 4, suggest that stratification and seed coat removal affect germination through their action on two different steps involved in the germination of the seed. They also suggest that embryo dormancy in *Pinus jeffreyi* is a relative con-



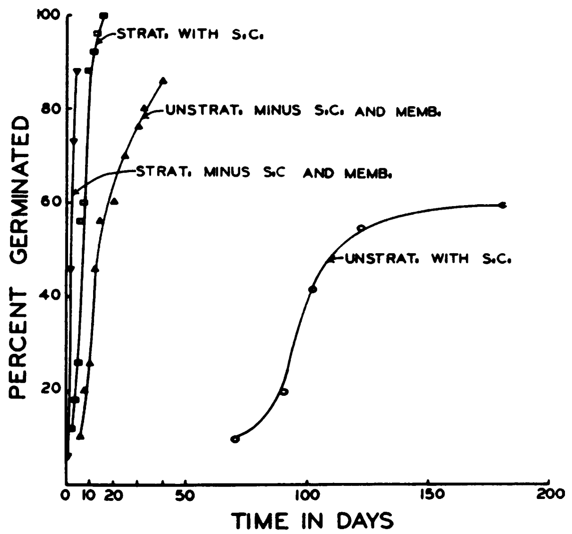
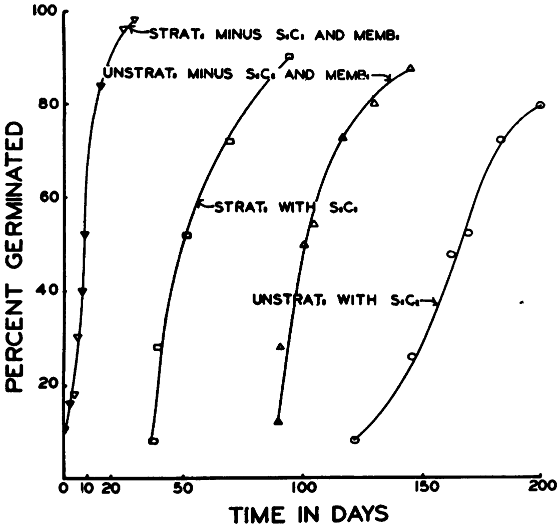


Fig. 1 (*top*). Germination at 25° C of stratified and unstratified seeds, with and without the seed coats and papery membranes. The number of days for 50 % of the seeds to germinate varied significantly (0.95 level) between treatments.

Fig. 2 (*bottom*). Germination at 15° C of stratified and unstratified seeds, with and without the seed coats and papery membranes. The number of days for 50 % of the seeds to germinate varied significantly (0.95 level) between treatments.



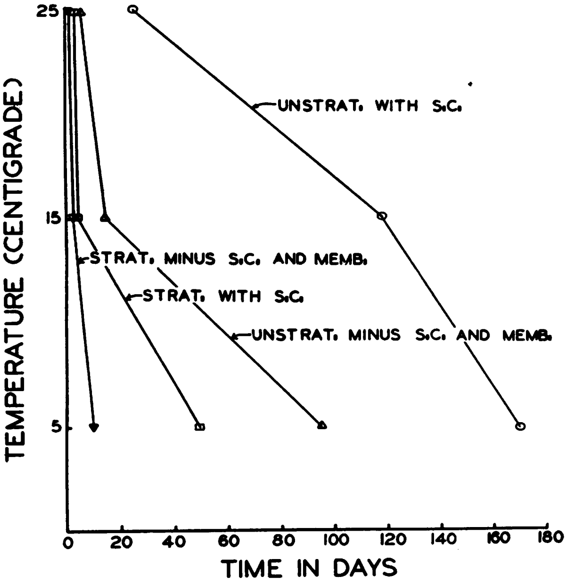


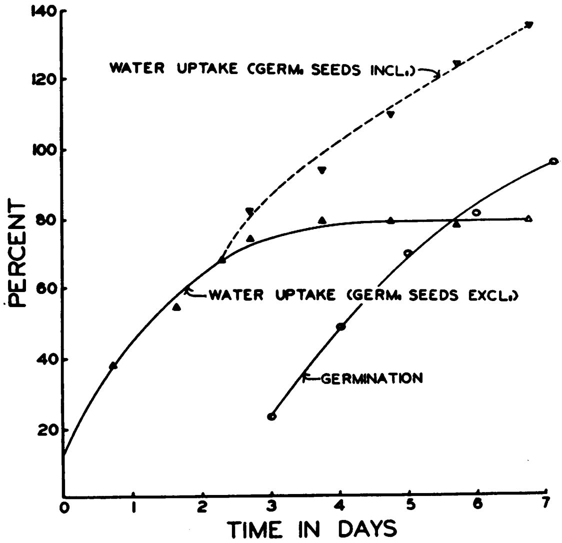
FIG. 3 (*top*). Germination at 5° C of stratified and unstratified seeds, with and without the seed coats and papery membranes. The number of days for 50 % of the seeds to germinate varied significantly (0.95 level) between treatments.

FIG. 4 (*bottom*). Average number of days for 50 % of the seeds to germinate at 5, 15 and 25° C. Stratified and unstratified seeds, with and without the seed coats and papery membranes are shown. Difference in treatments was significant (0.95 level) at all temperatues.

dition. Further speculation on these points, however, will be deferred until the effect of water uptake on germination is considered.

WATER UPTAKE OF SEED WITHOUT THE SEED COAT AND PAPERY MEMBRANE: At 25° C water uptake is closely associated with germination, with rapid water uptake being followed by rapid germination (fig 5). As a consequence, to the casual observer, it might appear that water uptake per se limits germination. However, such a conclusion is not justified inasmuch as the data do not allow one to specify what is cause and what is effect. This cannot be done unless the two processes can be studied separately.

Fortunately this can be accomplished by simply lowering the germination temperature to 5° C. At this temperature both water uptake and germination



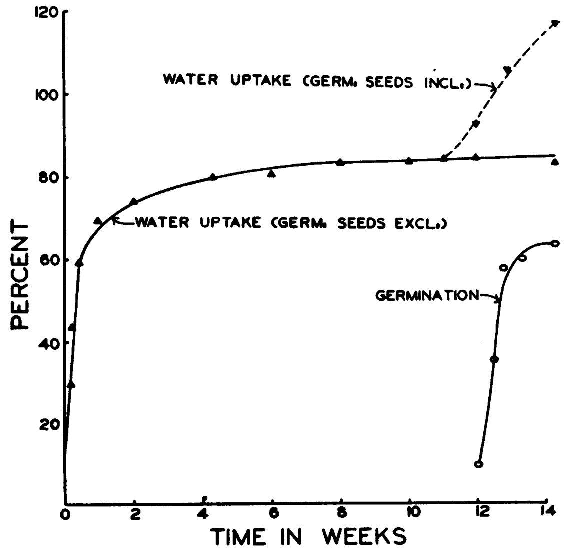


FIG. 5 (*top*). Germination and water uptake (% dry wt) at 25° C of unstratified seeds from which the seed coats and papery membranes were removed. Seeds were germinated on wet filter paper in closed Petri dishes.

FIG. 6 (*bottom*). Germination and water uptake (% dry wt) at 5° C of unstratified seeds from which the seed coats and papery membranes were removed. Seeds were germinated on wet filter paper in closed Petri dishes.

still occur. However, germination does not start for at least three months while water uptake starts immediately (fig 6).

At both 5 and 25° C there is a rapid uptake of water during the first 48 hours. After this initial period the seed continues to take up water but at a much reduced rate (figs 5 and 6).

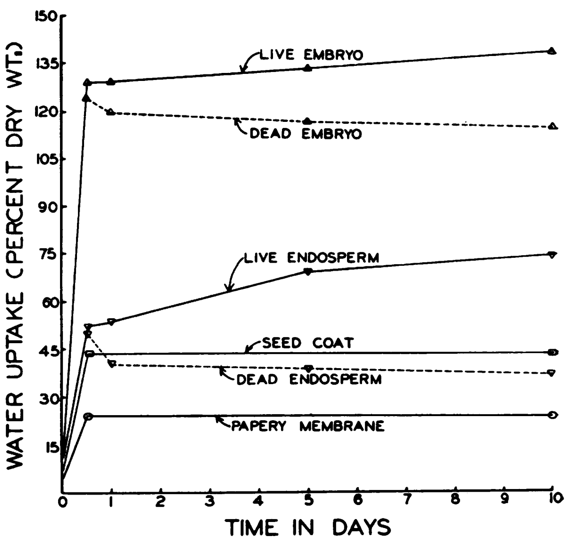
At 25° C the seeds have reached an average moisture content of 68 % after three days, at which point germination begins. In contrast, at 5° C the seeds take 7 days to reach the same average moisture content and germination begins 74 days after that (figs 5 and 6). Thus water uptake alone does not appear to control germination.

Each of the several tissues making up the seed has its own water uptake pattern. Therefore, to obtain a complete picture of water uptake, these individual tissues must be studied both separately and in close association. When each tissue is separated from the seed and placed on water-saturated filter paper water uptake of that particular tissue can be followed by subsequent weighings. When this was done each tissue showed a rapid initial uptake of water (fig 7). This was true for both the dead tissues of the seed consisting of the seed coat and papery membrane as well as for the live tissue consisting of the embryo and endosperm. Even when the endosperm and embryo tissue were killed by heat there was still a rapid uptake of water during the first 24 hours although this in turn was followed by a slight loss in water during the next 24 hours (fig 7).

It would thus appear that the initial water uptake by the seed is the result of imbibitional and osmotic forces. In the dead tissues of the seed only imbibitional forces appear to be involved. In the live tissues made up of the embryo and endosperm osmotic as well as imbibitional forces are indicated, first because the living cell normally contains considerable amounts of osmotically active substances and second because of the loss of water from the dead embryo tissue during the second 24-hour period (fig 7). Such a loss in water can only be explained if one assumes that the semipermeable condition of the cell membrane is destroyed when the tissue is killed by heat. Under such conditions the osmotically active substances would slowly diffuse out of the tissue into the surrounding water and as a consequence there would be a concomitant loss in water from the tissue.

After the rapid initial uptake of water by all the tissues—both dead and alive—the live tissues continue to absorb water at a reduced rate until the seed germinates (figs 5 and 6). This slow uptake is probably associated with a conversion within the cells of osmotically inactive substances such as starch to osmotically active substances such as glucose (5, 6). When the seed germinates there is again rapid uptake of water which is obviously necessary if the newly formed dividing cells of the embryo are to elongate.

At 25° C the water uptake pattern is essentially the same as at 5° C except that there is a telescoping of the time scale. Both curves show a sharp initial rise, followed by a gentle rise, followed finally by another sharp rise when the seed germinates (figs 5 and 6).



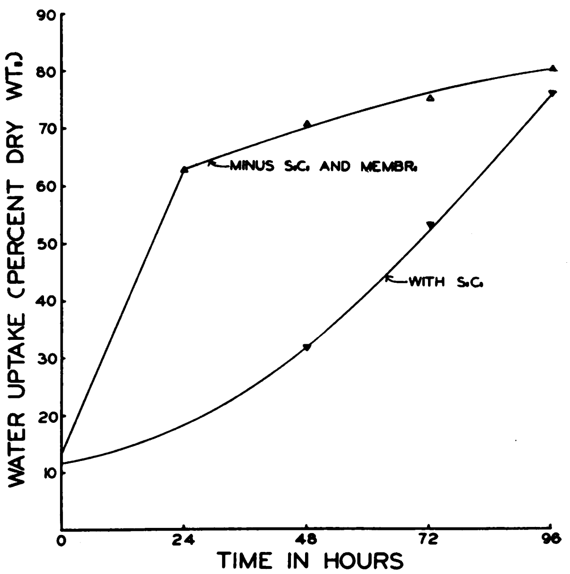


FIG. 7 (*top*). Water uptake (% dry wt) of the individual tissues making up the seed. The various tissues were held on wet filter paper in closed Petri dishes.

FIG. 8 (*bottom*). Water uptake (% dry wt) by intact seeds and seeds from which the seed coats and papery membranes had been removed. Seeds were in tap water at 5° C through which a stream of air was bubbled.

WATER REQUIRED FOR STRATIFICATION: Under normal stratification procedures the seed is free to continue water uptake throughout the period of cold treatment—three months in this case—which it does. This again complicates the interpretation of the relation between germination and water uptake. Is the slow continuous water uptake and resulting high moisture content responsible for the germination behavior of the stratified seed or is this behavior the result of chemical changes within the seed?

Fortunately again, this was easy to determine. Instead of providing a continuous source of water during stratification the seeds were soaked 48 hours in 5° C water through which air was continuously bubbled. Then the seeds were taken out, dried with a paper towel, and sealed in a number of large test tubes. These in turn were placed in incubators at 5, 15, and 25° C for three months. At the end of that time the seeds were removed, planted in wet vermiculite, and then placed in a 15° C incubator. Under similar conditions, seeds stratified by the usual process germinated readily (50 % in five days) while unstratified seeds required at least 118 days (fig 4). Of the treated seeds only those held for 3 months at 5° C had an appreciable germination in less than 60 days. Sixty percent of these seeds germinated in five days thus indicating that the seeds could indeed be stratified by this process. It is therefore apparent that all the necessary uptake of moisture occurred during the first forty-eight hours when the seeds were soaked (fig 8). After that, during the required remaining ninety days, chemical changes essential to rapid germination at 5° C apparently occurred provided the seed was kept at 5° C, but not if it was kept at 15 or 25° C. Thus again moisture per se does not appear to be limiting.

PERMEABILITY OF THE SEED COAT AND PAPERY MEMBRANE TO WATER: A comparison of water uptake by intact seeds and seeds divested of their seed coats and papery membranes, demonstrated the permeability of these two layers to water. When these seeds were soaked in 5° C water through which air was continuously bubbled, rapid water uptake did occur (fig 8). It is true that water uptake was more rapid when the seed coats and papery membranes had been removed. However, since the seeds still failed to germinate when the lag in water uptake was finally overcome, it would not appear that interference with water uptake per se is the factor limiting germination of *Pinus jeffreyi* seeds.

DISCUSSION AND CONCLUSIONS

From the data it would appear that a reasonable working hypothesis for further study of the seed dormancy mechanism in pine can be derived. Basically a two-step process is indicated, such as $S \rightarrow I \rightarrow G$ where S is the substrate, I is one or more intermediate compounds, and G is growth.

First let us examine this hypothesis in light of germination behavior at 5° C (fig 3). At this temperature removal of the seed coat did not in itself promote rapid germination. This only occurred when the seeds had been previously stratified. When the seed coats were removed from the unstratified seeds, germination did not begin for at least ninety days. From then on, however, germination was as rapid as if stratified seed had been used. This would be expected if intermediate compounds I accumulated in the unstratified seed during the initial ninety day period at 5° C. Thus it would appear that at 5° C the seed coat does not interfere with the accumulation of I, but does interfere with its utilization in growth.

Germination of stratified seeds at this low temperature was retarded unless the seed coats were removed. This suggested interference with either water uptake or gas exchange or both. Since the seed coat is permeable to water, uptake of water per se would not appear to be limiting. On the other hand, gas exchange is at least slowed down by the presence of the seed coat. This would tend to reduce the partial pressure of O_2 within the seed which in turn could be expected to interfere with certain oxidative reactions necessary for the utilization of material accumulated in the seed during stratification such as the postulated intermediates I.

Thus the proposed reactions $S \rightarrow I \rightarrow G$ in which I slowly accumulates during stratification and in turn is rapidly utilized for growth when the seed coat is removed is compatible with the data presented in figure 3.

Next let us examine the hypothesis in light of germination at 15° C (fig 2). Here again both seed coat removal and previous stratification at 5° C increased the germination rate. However, there was one principal difference. Germination at 5° C, even though the seed coats had been removed, took place only after sufficient time had elapsed for the seeds to become stratified—aproximately ninety days at 5° C for *Pinus jeffreyi*. At 15° C this was not true. Jeffrey pine seeds cannot be "stratified" at this higher temperature. Furthermore, removal of the seed coats from unstratified seeds, where $S \rightarrow I$ would not have been completed, speeded up germination by one-hundred days while removal of the seed coats from stratified seeds where $S \rightarrow I$ would already have been completed germination was only accelerated by five days. Thus it would appear that at this temperature, unlike at 5° C, the seed coat interferes primarily with the $S \rightarrow I$ step.

If at 15° C $S \rightarrow I$ goes on rapidly once the seed coat has been removed, what then can be the explanation for the retarding effect of the seed coat? Once again the partial pressure of O_2 in the seed must be considered. It is possible that in the presence of the seed coat and at this higher temperature, with the associated increase in respiratory activity, the partial pressure of O_2 in the seed is reduced to a critically low level where S is converted to other compounds C instead of being converted to I. If this occurred there might not be enough I accumulate for subsequent growth and germination, e.g., $S \rightarrow I \rightarrow G$. In effect
$\searrow C$
this would involve reactions of the same general type as those associated with the Pasteur effect (20). For additional speculation on the role of oxygen in the germination of dormant and stratified seed, the recent brief review article by Vegis (22) is suggested.

These data indicate the advantages of studying germination at different temperatures. The retarding effect of the seed coat can be studied at 15° C where it appears to interfere with the accumulation of intermediates. On the other hand at 5° C the effect of the seed coat on the utilization of the intermediates for growth can be studied. Also at 5° C the accumulation of intermediate I and its chemical identification can be most profitably studied.

Thus we have the basis from studies here reported upon for proposing a working hypothesis and also the basis for investigating its validity.

Summary

1. At 25 and 15° C removal of the seed coats and the papery membranes from unstratified *Pinus jeffreyi* seeds allowed rapid germination while at 5° C it did not. However, when the seed coats and papery membranes were removed from stratified seeds, germination was rapid at 5 as well as at 15 and 25° C. This was interpreted as indicating a condition of "relative" embryo dormancy which was overcome by stratification.

2. The seed coat and papery membrane surrounding the endosperm were found to be permeable to water. Furthermore, "stratification" was accomplished with only that amount of water absorbed by the seed during its first 48 hours in contact with water. Thus water uptake per se did not appear to limit germination.

3. Water uptake and germination were readily separable at 5 but not at 25° C.

4. The data suggested a convenient working hypothesis for the continued study of the seed dormancy mechanism in pine. In effect a two step process was indicated such as $S \rightarrow I \rightarrow G$ where S is the substrate, I is one or more intermediate compounds, and G is growth.

5. The effect of the seed coat on germination at 15 and 25° C suggests reactions of the same general type as those associated with the Pasteur effect.

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